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Short Communication

A new structured helical DPT-peptide containing a short canine adenovirus E4orf4 $PP2A_1$-binding sequence inhibits the PI3k survival pathway in human radio-resistant U87G glioblastoma cells

J. H. Colle and A. Garcia*

Laboratoire E3 Phosphatases, Unité RMN, Institut Pasteur, 25 rue du Dr Roux, 75015 Paris, France.

ABSTRACT

Radio-resistance of glioblastoma cells is a major challenge for developing an efficient therapy. Previous studies have established that constitutive activity of the PI3K-Akt pathway and radio-induction of cellular senescence are involved in radio-resistance of U87G cells. Herein, using a rational molecular design, we identified a new potential anti-tumor DPT-peptide, named DPT-$E4orf4_{23-38}$, that combines the HIV-1 Tat shuttle plus the canine adenoviral type 2 $E4orf4_{23-38}$ $PP2A_1$-binding sequence. This new chimeric sequence is a helical peptide that inhibits the survival of U87G cell line. In addition we found that DPT-$E4orf4_{23-38}$ inhibited Akt-phosphorylation (ser473), and decreased the growth of highly radio-resistant U87G cells. Finally DPT-$E4orf4_{23-38}$ also decreased the growth of X-irradiated (2Gy) U87G senescent cells. Together our results suggest that DPT-$E4orf4_{23-38}$ alone or combined with irradiation might be clinically evaluated as a potential therapeutic molecule against PI3K-dependent and human radio-resistant and radio-senescent glioblastomas.

KEYWORDS: radioresistance, glioblastoma, structured DPT-peptide

INTRODUCTION

PP2A is a tumor suppressor frequently inhibited in human tumors that represents a druggable target against cancer [1]. Interestingly, the trimeric ABC PP2A holoenzyme named $PP2A_1$ can inactivate Akt, and $PP2A_1$ inhibition activates tumor survival pathways associated with cancer progression [2]. We have previously proposed that specific targeting of PP2A holoenzymes by viral proteins is a general biological strategy used by various viruses to specifically deregulate intracellular pathways of their hosts [3]. Interestingly, several studies have established that human Ad2 E4orf4 protein interacts with the regulatory B subunit of trimeric PP2A holoenzyme ABC, named $PP2A_1$, to specifically induce p53-independent death of human cancer cells [4, 5]. In addition E4orf4 is also a specific cancer killer that displays no toxic effects in primary human cell types derived from distinct tissues [6].

To specifically deregulate a PP1/PP2A-dependent pathway, we have previously proposed [7] and extensively characterized [7-10] the drug phosphatase technology (DPT) concept involving the design of PP1/PP2A-interacting pro-apoptotic cell-penetrating peptides named DPT-peptides. In this regard we have previously shown that $E4orf4_{23-38}$ sequence of canine adenovirus E4orf4 protein is a $PP2A_1$-binding and non-penetrating sequence [10].

The constitutive activation of the PI3K-Akt pathway occurs in a majority of human glioblastomas [11]. We recently found that FTY720 and DPT-Cog, two PP2A pharmacological activators, downregulated the $PP2A_1$-dependent PI3K/Akt survival pathway

*Corresponding author: agarcia@pasteur.fr

and also inhibited survival of highly radio-resistant U87G human glioblastoma cells [12, 13].

In this study using human glioblastoma U87G cells, we showed that DPT-E4orf4$_{23-38}$, a bipartite-structured cell-penetrating sequence containing Tat shuttle fused to the PP2A$_1$-binding canine adenoviral E4orf4$_{23-38}$ sequence, can deregulate a constitutively active PI3K-Akt pathway in these cells. In addition our results suggested that DPT-E4orf4$_{23-38}$ can overcome a senescence-mediated resistance mechanism induced by irradiation.

MATERIALS AND METHODS

Cells

We used the U87G glioblastoma cell line (kindly gift by Pr. Marie Dutreix, Curie Institute, Orsay). The cells were maintained in Dulbecco's modified Eagle's medium (Gibco) with 10% fetal bovine serum, without any antibotic and incubated at 37 °C in a humidified atmosphere of 5% CO_2.

Peptides and pharmacological agent

High-performance liquid chromatography-purified NH_2–biotinylated peptides (purchased from PolypeptideGroup) were prepared by solid-phase peptide synthesis and then dissolved in dimethyl sulfoxide (DMSO) and stored at -20 °C pending use. The sphingoid FTY720, used as a positive control of PP2A activation, was from Euromedex.

Circular dichroism (CD) measurements

CD spectra were recorded on an Aviv 215 spectropolarimeter (Aviv Biomedical) and data were normalized to the molar peptide bond concentration and path length and expressed as mean residue ellipticity ([θ] degree cm^{-2} $dmol^{-1}$) as previously described [10].

Cytotoxicity assays

A total of 3,000 cells were incubated for 24 hours with different concentrations of pharmacological agents. Cell cytotoxicity was analyzed by a colorimetric assay using 3-(4, 5-dimethylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide (called MTT) for adherent cells as described by the manufacturer (Sigma).

Western blot analysis

Exponentially growing cells (10^5 cells) were seeded overnight in 24-well culture cell plates, in sub confluent monolayer, prior to pharmacological treatments. For extract preparation, cells were rinsed in cold phosphate buffered saline (PBS), scraped, pelletized and lysed in Thermo Scientific buffer (89900), supplemented with a cocktail of Halt protease and phosphatase inhibitors (78442), according to the manufacturer instructions, and finally sonicated for 2 min. at 50% pulse.

The protein concentration in each sample extract was quantified using the Bio-Protein assay, Bio-Rad Laboratories. Lanes were loaded with the material corresponding to 20-40 µg of cell protein extract. The following primary antibodies were used: anti-Phospho-AKT (Ser 473) (D9E), and anti-AKT (pan) (C67E7) from cell signaling technology and also anti-HP1γ (2MOD-1G6), from Euromedex. In addition goat peroxydase-labeled anti-rabbit IgG or horse peroxydase-labeled anti-mouse IgG from VECTOR Laboratories were used as secondary antibodies. Immunoreactive proteins were revealed using PIERCE ECL western blotting substrate.

RESULTS AND DISCUSSION

DPT-E4orf4$_{23-38}$: a new structured helical DPT-peptide that can inhibit PI3K-dependent survival of U87G cells

We have previously published that Tat-E4orf4$_4$, containing the sequence YGRKKRRQRRRSMLE SPMEFLFD, is an unstructured and biologically inactive peptide that combines Tat-penetrating sequence and E4orf4$_{27-38}$ sequence [10]. In this study we first applied the DPT-technology to generate DPT-E4orf4$_{23-38}$, a new E4orf4 mimetic peptide containing the 16 PP2A$_1$ binding residues of the canine type 2 E4orf4$_{23-38}$ sequence fused in its N-Ter part with the TAT shuttle sequence (for sequence see figure 1 upper panel). Interestingly, in contrast to unstructured Tat-E4orf4$_4$, we found that DPT-E4orf4$_{23-38}$, containing an additional N-Terminus E4orf4$_{23-26}$ (RELG) sequence (see figure 1 upper panel and), required for PP2A$_1$ binding [10], exhibited a profile with a maximum peak at 220 nm, which is consistent with the presence of a predominantly extended helical structure (Figure 1 lower panel).

Thereafter using MTT assay we examined the cytotoxic effect of DPT-E4orf4$_{23-38}$ in the U87G glioblastoma cell line. As shown in figure 2A,

Origin of peptides containing Tat and PP2A$_1$ interacting sequences

Protein of origin	Acronym	Sequence
Tat	TAT	YGRKKRRQRRR
Canine Ad2 E4orf4$_{23-38}$	E4orf4$_{23-38}$	**RELG**SMLESPMEFLFD
Tat+Canine Ad2 E4orf4$_{27-38}$	Tat-E4orf4$_4$	YGRKKRRQRR----SMLESPMEFLFD
Tat+Canine Ad2 E4orf4$_{23-38}$	DPT-E4orf4$_{23-38}$	YGRKKRRQRR**RELG**SMLESPMEFLFD

-A single letter amino acid code is used for all peptides; RELG motif is in bold.

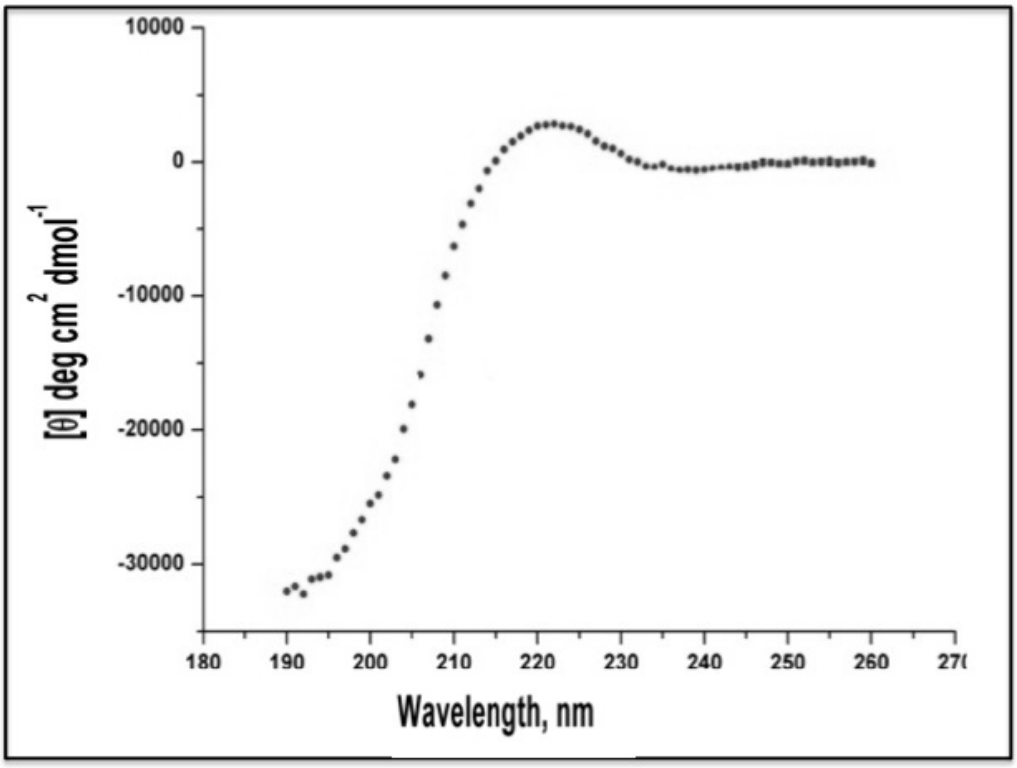

Figure 1. Structural analysis based on CD spectra of DPT-E4orf4$_{23-38}$ peptide.

- Sequences of Tat and DPT-peptides are shown in the upper panel.

- The lyophilized DPT-E4orf4$_{23-38}$ was re-suspended with 20 mM Tris-HCL (pH 7.5) at a concentration of 65 μM and used for CD analysis as previously described [10]. The result is shown in the lower panel.

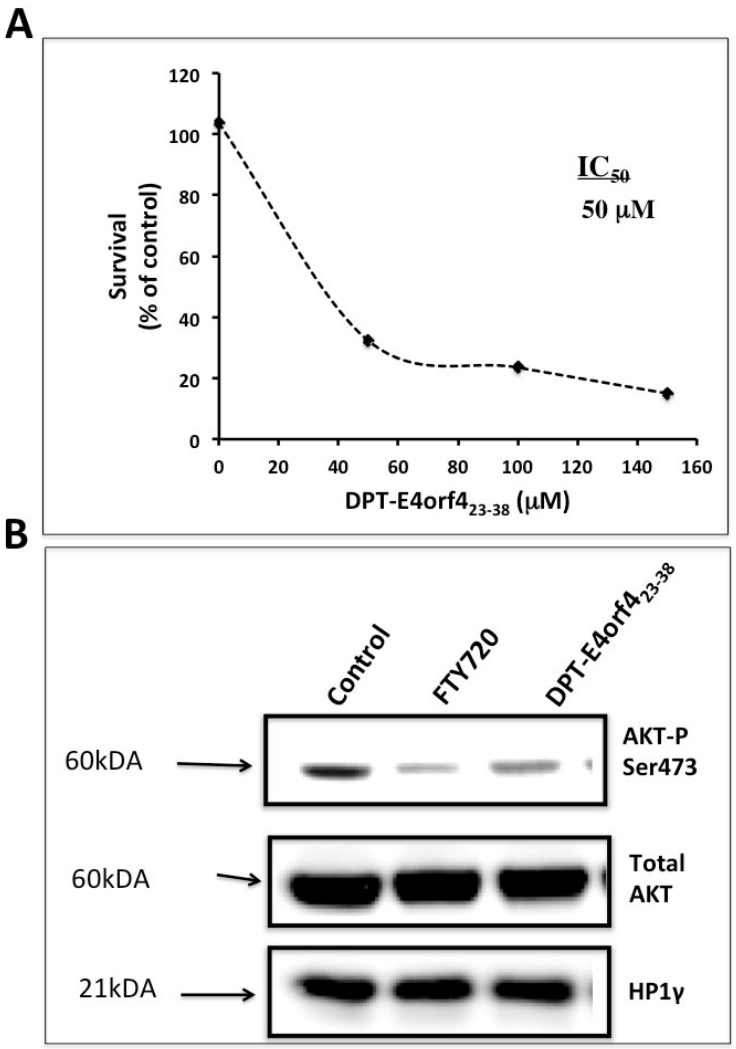

Figure 2. DPT-E4orf4$_{23-38}$ inhibits a constitutively active PI3K/Akt survival pathway in U87G cells.

(A) U87G cells were incubated for 24 h with various concentrations of DPT-E4orf4$_{23-38}$ and cell viability was analyzed by MTT test (n = 3).

(B) U87G cells were untreated (negative control) or treated for 5 h with DPT-E4orf4$_{23-38}$ (150 µM) or 1 h with 50 µM of FTY720 (used as a positive control for AKT-dephosphorylation) and the effects of DPT-E4orf4$_{23-38}$ and FTY720 on PI3K/Akt survival pathway were analyzed by western blot monitoring of Akt-phosphorylation using a specific Akt473 antibody analysis. The same blot was re-probed with a monoclonal antibody to total Akt and with an antibody to HP1γ as internal control.

we found that a 24 hr treatment of the U87G glioblastoma cells, with increasing concentrations of DPT-E4orf4$_{23-38}$ resulted in a dose-dependent reduction in cell viability (estimated inhibition rate IC_{50} = 50 µM).

In addition, and in consistence with the previously described cell survival dependence mediated by a constitutive activity of PI3K/Akt pathway in U87G glioblastoma cells [12], western blot analyses showed that DPT-E4orf4$_{23-38}$ also decreased Akt phosphorylation (ser473) in U87G cells (Figure 2B). Together these results indicated that DPT-E4orf4$_{23-38}$ counteracted the constitutively active PI3K/Akt pathway that is required for survival of highly radio-resistant U87G glioblastoma cell line.

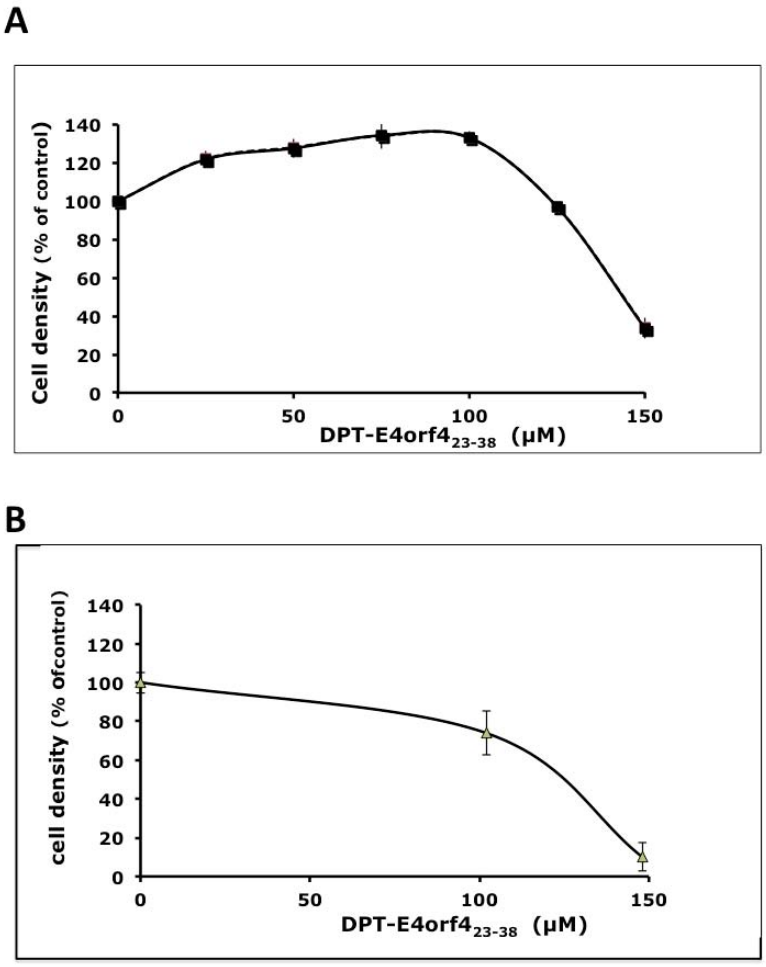

Figure 3. Effects of DPT-E4orf4$_{23-38}$ pharmacological treatment on cell growth.

(A) Cell growth curves of U87G cells after DPT-E4orf4$_{23-38}$ treatment.

48-well microplates were seeded (n = 4) with 2 x 10^4 cells for U87G and 18 hours later cells were treated with different doses (0-150 µM) of DPT-E4orf4$_{23-38}$; 72 hours later cell densities were measured.

(B) Cell growth curves of U87G cells following exposure to X-ray radiation and DPT-E4orf4$_{23-38}$ treatment.

24-well microplates were seeded (n = 4) with 10^4 cells for U87G. As previously described [12], the cell densities were recorded in the culture of U87G X-irradiated cells (2Gy), cultured for 4 days, then treated for 24 h with DPT-E4orf4$_{23-38}$ (0-150 µM).

Effect of DPT-E4orf4$_{23-38}$ on growth of U87G glioblastoma cells

As illustrated in figure 3A, treatment with increasing concentrations of DPT-E4orf4$_{23-38}$ for 72 hrs resulted in a dose-dependent reduction in cell density in the U87G glioblastoma cells. Furthermore, similar inhibitory effects were observed when U87G cells expressing SA-β-Gal senescence marker induced by X-ray radiation (2Gy) [12], were cultured 4 days prior to DPT-E4orf4$_{23-38}$ treatment (Figure 3B).

CONCLUSION

This study illustrates that, in contrast to the previously characterized unstructured Tat-E4orf4 [10], treatment with the DPT-E4orf4$_{23-38}$ helical

peptide, containing the additional canine E4orf4 RELG motif, i) down-regulated the constitutively active PI3K/Akt survival pathway, and ii) inhibited survival and proliferation of non-irradiated or irradiated radio-resistant and radio-senescent U87G cells.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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